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The Effects of Phosphate on the Metamorphosis of Larval Western Barred Tiger Salamanders (Ambystoma mavortium)

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The Effects of Phosphate on the Metamorphosis of Larval Western Barred Tiger Salamanders (*Ambystoma mavortium*)

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Abstract

This investigation will collect data to assist in determining if elevated aquatic phosphate levels affects the metamorphosis rate of larval western barred tiger salamanders (*Ambystoma mavortium*). Monoammonium phosphate fertilizers are being used on crops in Nebraska (NDA, 2017). This area lines up with the area the western barred tiger salamanders are disappearing from (Damme, 2018). Monoammonium phosphate is made up of nitrogen and phosphate. There have been several studies showing how nitrogen is harmful to amphibians such as this salamander (Griffis-Kyle, 2007) (Griffis-Kyle & Richtie, 2007), but there have not been many showing how phosphate affects amphibian's metamorphosis in the aquatic environment. Therefore, this investigation will look at how elevated levels of phosphates affects the metamorphosis of larval salamanders. This study will incorporate a control and treatment group of salamanders that will be followed through their metamorphosis. The data was statistically analyzed. After analyzing the data, it was found that only one of the two treatment salamanders showed a correlation between the phosphate level and its metamorphosis. Data obtained did not significantly indicate the effects of elevated phosphate levels on Western salamander metamorphosis.

Key Words: metamorphosis, phosphate, western barred tiger salamander (*Ambystoma mavortium*), conservation, fertilizer

Dedications/Appreciations

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Introduction

This study will examine the effects of elevated aquatic phosphate levels on the larval stage of western barred tiger salamanders (*Ambystoma mavortium*). This is important as the southeastern NE population of these salamanders has been declining. The areas of decline line up with this fertilizer's usage as shown in Damme's study during 2018. Specifically, this study investigates the effects of the phosphate component of the fertilizer on the metamorphosis rate of the larval salamanders. The salamanders begin their life cycle in the water as eggs. When they hatch they are in their larval form. In the larval form, they have gills and are unable to leave the water. As they mature into the terrestrial stage they lose their gills and gain the ability to leave the water. Many different things can affect the salamander in their larval stage. When they are dependent on water to survive, they are more vulnerable to a wide range of environmental impacts. Runoff from fertilizers is an increasing problem to many ecosystems. Runoff is when water washes down into a large body of water. On the way to the large body of water, it may pick up any forms of particulates including things such as soil, leftover fertilizer, or herbicides. The larval form of the *Ambystoma mavortium* depends on the water to survive.

These salamanders are native to Nebraska. Previously, they were found all across the state, but in the past 10-12 years the salamanders have been declining in Cass, Douglas, Gage, Jefferson, Johnson, Lancaster, Nemaha, Otoe, Pawnee, Richardson and Washington counties (Ferraro, 2016). There they share some of their habitat with the small-mouth salamander (Ferraro, 2018). The western barred tiger salamander population has been absent from this specific area and no direct threat has been determined. This study will help to determine a possible threat to the population. Agricultural fertilizers and pesticide usage are thought to be a problem for *Ambystoma mavortium*, but there has not been confirmation due to a lack of information. If the fertilizers in question are found to be detrimental to the health of *Ambystoma*

mavortium, then perhaps this information can be used to find a different method of fertilization that does not harm the salamanders. Additionally, this research can help detect possible environmental problems in the area as amphibians are particularly sensitive to changes in their environment.

In a similar situation, there was a Western barred tiger salamander die-off in Canada (Ashpole, 2011). This die-off, in particular, was unexpected as they were taking an assessment of the salamander population, looking to gain a population estimate for the species. The assessment was to determine the population size of the western barred tiger salamanders in different sub-habitats. This is important as the salamander is endangered in Canada. Ashpole (2011) proposed several theories that may be why the salamanders died, such as, the use of pesticides, presence of ranavirus, introduction of goldfish, and/or the low immigration potential of salamanders from other ponds. The pond in question was located near farming activities where they applied pesticides (Ashpole, 2011).

In a different area, *Ambystoma mavortium* living in the Southeastern High Plains of Texas were found to have, on average, smaller body sizes when living in close proximity to agriculture compared to populations living in grassland areas (Gray and Smith, 2010). Their study was observational. They took data on the body size of the salamanders and frogs over the course of two years. They used drift fences and pitfall traps to catch the subject species. The overall trend shows that the amphibians in areas near agriculture tended to have a smaller body size. This may imply that salamanders living in areas around agriculture may have a stunted growth rate. The salamanders that are disappearing from Nebraska live near agricultural areas, so that may be a factor negatively impacting them. The stunted growth rate could manifest itself in the form of a slowed metamorphosis or slowed weight gain.

The research being conducted may also be applied to other conservation efforts. All animals in the ecosystem have their own purpose and effects on their surroundings. Although, not all animals and plants are well documented on their benefits to the ecosystem they are all important pieces of their environment. Amphibians, in particular, are highly important to conservation scientists. The study species, the Western Barred Tiger Salamander, is an amphibian. Amphibians play an important role as an indicator species. An indicator species is one that is affected by changes in the environment before other species. They act as a canary in a coal mine, serving as a sign that something is wrong with an ecosystem which may negatively affect other species. Since amphibians absorb oxygen and water through their skin they are particularly susceptible to chemical changes in their environment. In areas that amphibians are doing well, the environment should be relatively stable. Comparatively an area where amphibians are disappearing may indicate that the environment may not be stable or able to support the ecosystem.

Monoammonium phosphate was found to be used in the area the salamanders were located (Damme, 2018). Ammonia is a chemical compound containing nitrogen. One of the ammonia hotspots is located on the Southeast portion of Nebraska (Damme, 2018). This research project found that ammonium phosphate fertilizers are being used in the Southeastern portion of the state. Since the fertilizer is applied directly to the land, it should have a relatively equal chance of affecting larval and terrestrial forms of *Ambystoma mavortium*. Because the fertilizer should have an equal chance of affecting both life stages, the next step would be to determine how the fertilizer is affecting the larval forms. This research can also be used to predict the effects of this fertilizer on other native amphibians, such as the ten species of anurans, and the small-mouth salamander (*Ambystoma texanum*) (Ferraro, 2018).

There have been no direct studies of how phosphate fertilizer affects *Ambystoma mavortium* specifically. Despite this, nitrogen in the form of nitrite has been shown to cause changes in egg hatching time of salamanders (*Ambystoma mavortium*) (Griffis-Kyle, 2007). Additionally, nitrite has also been found to cause slower development in larval salamanders (Griffis-Kyle, 2007). This slowed development was shown in the salamanders hatching before they had developed proper respiratory functions (Griffis-Kyle, 2007). In a different study, the presence of nitrites was not found to have a significant effect on *Ambystoma mavortium*'s growth or development (Griffis-Kyle and Richtie, 2007). However, it was found that nitrites in the wood frog's (*Rana sylvatica*) ecosystem negatively affected their development (Griffis-Kyle and Richtie, 2007). It is from this study, showing that a form of nitrogen slows the salamander's growth rate, that spurred me to look into the effects of monoammonium phosphate on the salamander's metamorphosis rate.

The components of the fertilizer, nitrogen and phosphorus, have been shown to have negative impacts on the environment when in high amounts. In a study by Neesteson, when the phosphorus and nitrogen input exceeds the output the chemicals can leach into waterways. When the chemicals do this, it can negatively impact drinking water and can cause algal blooms (Neesteson, 2000). These blooms can cause the death of fish and other organisms commonly found in and around the water due to the loss of oxygen in the water. Neesteson also mentions that NH_3 volatilization can contribute to creating acid deposition and N_2O emissions play a role in climate change and deterioration of the ozone layer.

This current research is a case study looking into the phenomenon of the disappearance of the salamanders in the Southeastern portions of the state. This study is specifically looking at the effects of phosphate on larval salamanders. There have been several studies looking at the effects

of nitrogen on the salamanders. Since the fertilizer is composed of both nitrogen and phosphate, tests will be done to determine how a higher phosphate level will affect the salamanders. One group of four salamanders will serve as a control group and the other four salamanders will have the same amount of sodium phosphate added to their tanks. Based on Griffis-Kyle's research, I hypothesized that additional phosphate in the water will cause the larval salamanders to have a slower metamorphosis rate than the control group with no additional phosphate. A mixture of qualitative and quantitative data will be used. The qualitative data will come from observed effects of the fertilizer, which may be but not limited to, changes in behavior, changes in appearance, and the possibility of death. Quantitative data will come from recording data on the salamanders such as amount of food eaten, temperature, pH, hardness and phosphate levels in the tanks.

Two questions that pertain to the research project are as follows: How does elevated phosphate levels affect the metamorphosis rate of the larval salamanders? How does the phosphate affect the larval salamanders differently than the terrestrial ones? To address these questions, data will be recorded for the days it takes the salamanders to morph, what stage they are at once the fertilizer has been in their tank, and what water levels are reduced to induce metamorphosis. Differences in the salamander's behavior and/or appearance in relation to the fertilizer will be recorded.

Materials and Methods

This research is a case study, looking at the specific phenomenon of presence of phosphate in relation to metamorphosis. This is similar to Griffis-Kyle's study looking at how different forms of nitrogen affect the salamanders. In their study they tested different levels of sodium nitrite on the salamander larvae and followed some of them through their

metamorphosis. A similar setup will be created, but instead one treatment level of phosphate fertilizer will be tested in addition to a control group. The salamander larvae will also be separated for the duration of the study. Due to the fact that the salamander larvae need to be separated, they will require their own individual tanks. We do not have enough room to support more than 8 tanks, so there will be 8 salamanders in the study. Additionally, the larvae will be observed as they progress from larval to terrestrial form.

In order to determine if the phosphate levels affected the metamorphosis rate of the salamanders the phosphate level in each tank was recorded weekly alongside other baseline data. The other baseline data consisted of the amount of food eaten, temperature of the water, pH, hardness, and when the salamander morphed.

The salamanders were allowed time to adjust to their tanks for about a month and three weeks. During this time, variables such as feeding habits and the temperature of the water began to be recorded. These are key variables to prevent the salamanders from morphing before the phosphate is added. Temperature is an important factor, as the colder it is, the less likely the salamanders are to metamorphosize. The total food eaten by the salamanders is also important because if the salamander feels it is not being fed enough it will metamorphose to change its feeding habits. This happens naturally in their natural habitat because it reduces competition amongst the larvae and allows the terrestrial salamanders to eat different food. Until the phosphate is in the tanks, the salamanders should remain in a larval form, so it can be assessed how the phosphate affects their rates of metamorphosis.

In order to determine the concentration of phosphate used several studies were consulted. One study found that when nitrogen and phosphorus fertilizers were used, the phosphates were usually in smaller supply than the nitrogen (Sharpley, 1995). They also determined that different

types of manure had different concentrations of phosphorus (Sharpley, 1995). The other study found that the USEPA phosphorus limit for streams that enter lakes is a concentration of .05 ppm or lower (Mullins, 2009). This study also found that the USEPA recommended concentration of phosphorus in flowing streams is 0.1 ppm or lower. They also found that the concentration of phosphorus in the soil increases with increasing treatment levels. This information is shown in figure 5 of their research. The highest accumulation of phosphorus was in areas where the annual application was 200 pounds per acre (Mullins, 2009). This is notably lower than the label rate for monoammonium phosphate ranges from 871 pounds per acre to 4,356 pounds per acre. Due to this study it was determined that using a concentration of .05 ppm is best to see if the current recommendations for lakes are still harmful to the Western Barred Tiger Salamander.

Some limitations of this study are that the salamanders cannot be in the same tank. If they are in the same tank they will bump into each other more often, thus being more likely to metamorphosize. In order to make sure all possible morphing variables are accounted for, the salamanders have their own tanks. This makes the sample size smaller, as there is not enough space to support 16 tanks with individual salamanders in them. Another limitation is having the room maintain a temperature cold enough to keep the salamanders from morphing. There is a different research project going on in the same room which makes it, so the room must be warm enough to support them. Due to this ice packs have been added to each of the salamanders' tanks to keep them cold enough to prevent morphing.

Another limitation was finding a good method to measure and maintain the phosphate levels in the tanks. The first method using an electronic reader was relatively consistent, but it ran out of phosphate testing strips. A new system using vials and color matching to determine the concentration of phosphate was used for several weeks after the first test ran out of strips. After

the second phosphate testing method was used, it was found that the phosphate levels fluctuate dramatically. Larva #4's tank went from concentration of 8 ppm, to a concentration of 10 ppm the next week, and a concentration of 5 ppm the week after. It did not make sense that the concentration of phosphate decreased from 10 ppm to 5 ppm since phosphate was added before the tank was tested and found to be at 5 ppm. A third way of measuring the phosphate was found utilizing a test kit by LaMotte that can measure concentrations of phosphate from 1 ppm up to 100 ppm. This test was consistent in measuring the phosphate levels, so it was used for the remainder of the study.

The salamanders will have changes made to their tanks once the phosphate level is stable to encourage metamorphosis. Until the other animals in the lab come out of hibernation the main part of the lab will be kept cold which may help keep the salamanders from morphing. To induce metamorphosis the ice packs will be removed from the water to allow the salamanders to have a warmer water temperature. After they adjusted to the warmer temperature of the water, the water level was slowly decreased as the water evaporated. While the water level was decreasing no new water was added, until it got below the height that allowed the filter to function. When the water level got that low, only enough water to allow the filter to function was added. As the water level decreases the salamanders will be pressured to morph more. Throughout the whole process the concentration of phosphate will be kept the same in all the tanks. When a tank was low on phosphate, more phosphate was added to maintain the concentration. Additionally, to induce metamorphosis the salamanders will go from a daily feeding schedule to a decreased feeding schedule. This prompts them to morph in order to find a new food source. After the salamanders have morphed the data collected will be analyzed to determine the effect of phosphate on their metamorphosis.

Collection

To begin the study, salamanders were collected. The salamanders were obtained from Bessey National Forest in Thomas county NE, on September 14, 2019. To take the salamanders from the small ephemeral ponds in the prairie, a small seining net was utilized. This net was utilized so that the whole area of the small pond could be covered and as many salamanders as possible could be obtained. The seining nets are similar to dip nets. Dip nets were found to have no bias between using them or funnel traps to catch salamanders (Dana, 2010). Since there was no bias found in the trapping method of using dip nets it is assumed that a representative sample of the salamander population was found when using the seining nets.

The seining nets were able to catch many animals in the pools including some leopard frogs. Using a seining net is similar to using a dip net, only that the seining net is bigger and has larger holes in the net. The larger holes were not a disadvantage, as the salamander larvae are larger than the holes. The seining net used required at least two people. One person to hold one end of the net in a set place. The other person to pull the net as far as it can get from the other end. Then, the people adjust to allow the weighted net bottom to settle across the bottom of the pond. The person on the moving side then slowly moves toward the other side of the pond. When both people are in a line with each other they both advance to the end of the pond. Once they make it to the end the net is carefully pulled out of the water and examined for animals.

When examining the net, if a larval salamander was found it was placed in a bucket full of the water it had previously been in. Enough buckets were brought for the salamanders to be about 10-15 individuals per bucket. Each pond was seined multiple times to ensure that enough salamander larvae were found for the studies being done in the lab. After seining three ponds, enough salamanders were obtained. The ponds are in Thomas County NE located at N

41°52.395', W 100°26.642'; N 41°15.238', W 100°28.013; and N 41°49.392', W 100°27.329'. We obtained 15, 24, and 2 larvae from each pond respectively. In total 41 larvae were caught. The larvae were then transported back to the herpetology lab on UNL's East Campus. Once the larvae were in the lab, they were placed in a large tank to hold them until the smaller tanks were ready for them.

Setup and Data Quality Assurance

After five days of being in the lab, some of the larval salamanders were found to be morphing into the terrestrial form. This process was not expected to occur until later in the study. In order to combat this two large ice packs were placed in the large tank with all the larvae in it on November 19, 2019. These ice packs were changed twice daily until September 26, 2019 when their individual tanks were set up. The ice packs make the water cooler, which makes the salamander larvae less likely to expend the energy to metamorphosize into the terrestrial form.

The individual tanks were placed on a shelving unit in the procedure room in the lab to keep them separate from the rest of the lab. The initial plan was to have two salamander larvae per tank. After thinking about how the larvae were already trying to morph, it was realized that the more the larvae touched each other, the more likely they were to morph. In an effort to keep morphing to a minimum the larvae were each given their own tank. In total there was enough room for eight tanks, so eight larvae were selected for the study. The tanks were placed on a shelving unit with 4 on the top shelf and 4 on the bottom shelf. Keeping the larvae in separate tanks was one of the ways the quality of data was ensured. The larvae were picked based on the length of their gills. The larvae with longer gills were selected as when larvae begin to morph their gills shrink. The larvae with longer gills were identified by sight.

Each tank was provided with a thin, equal layer of gravel at the bottom of the tank. To further ensure the quality of data the following actions were taken. The gravel was cleaned to ensure there were no chemicals on the rocks. A filter was placed in each tank to oxygenate the water, and to help clean out the waste material from the larvae. No charcoal was placed in the filters, as to avoid a change in the concentration of the chemicals in the water. After placing the filters in the tank, the tanks were filled up to above the area of the filter that sucks in the water. Each tank ended up with 20 liters of water. The water used in the tanks comes from a natural stream in order to avoid contaminants that may be in tap water. After filling each tank to 20 liters a line was drawn on the tank to show where the 20-liter mark is, so the water can be kept at the right level. The tanks were left to sit for two days to ensure that the water was flowing well in regard to the filter. On September 26, 2019 the salamanders were placed in their tanks. Each tank was given a number 1-8 which was indicated on the tank. Numbers 1-4 were on the top shelf while numbers 5-8 were on the bottom shelf. Once the salamanders were placed in the tanks a small ice pack was placed in each of their tanks to slow the metamorphosis rate. These ice packs were changed once a day until December 13, 2019.

Tank Adjustments

The salamanders were then given five weeks and five days to settle into their tanks before adding the phosphates. Prior to adding the phosphates, the temperature both before and after changing the ice packs was recorded for about 3 weeks. This was recorded to ensure that the ice packs were changing the temperature of the water and that the water was not too cold or warm for the salamanders. In addition to the temperature, the feeding habits of the larvae were also recorded. This was recorded to ensure that the larvae did not become ill or have health problems related to eating. The larvae were also observed daily. During the daily observations before the

phosphate was added larvae #3 and #8 began morphing. Since it was still before we had added the phosphate to the larvae we switched the morphing individuals with different larvae that had large gills.

To determine the necessary concentration of phosphate to use, a literature review was completed. In Mullins's study it was found that the EPA allows a concentration of .05 ppm of phosphate in the water that flows into a lake. Based on this data it was decided that this concentration of phosphate would be tested. Prior to adding the phosphate, the levels of phosphate in the tanks were recorded to ensure there were no background chemicals. The first method of measuring phosphate utilized an electric phosphate meter that recorded the level of phosphate in a water sample. This worked well, however, there were only three strips left to use this method. The rest of the strips had been used the year prior while researching the terrestrial salamanders. Due to this, new phosphate testing kits were obtained to measure the amount of the chemical in the water. They were used several times prior to adding the phosphate. Despite the multiple tests and not changing anything about the water it was found that the phosphate levels fluctuated drastically. Due to this high fluctuation a new phosphate test kit was found. This test began usage on November 19, 2019. The kit is by LaMotte; it utilizes a colored bar on the side which matches to the solution created from the sample water. If a tank was found to be at a concentration of phosphate equal to 10 ppm, a 1-10 dilution was then made to determine if the tank had a concentration above 10 ppm or if the concentration was only at 10 ppm.

Adding Phosphates

Phosphates were first added on November 5, 2019. They were added to create a .05 ppm concentration of phosphorus. First, a tray was placed onto a scale and tarred. After the tray was tarred a scalpel was used to scoop out .05 grams of sodium phosphate. After the .05 grams of

sodium phosphate was obtained it was mixed in a 1-liter solution of natural river water in a beaker. To mix the solution thoroughly the beaker was placed on a Vortex Genie 2. The solution was mixed for 1 minute on level 3. After this 40 ml of water was taken from the control tank and was placed in a 250 ml beaker. Then 20 ml of the 1-liter phosphate solution was also placed in the 250 ml beaker. The 250 ml beaker was then mixed on the Vortex Genie 2 for 1 minute on level 3. The 250 ml beaker solution was then poured into the tank by the filter. These steps were repeated for all four of the treatment tanks.

After adding the phosphates, the second phosphate test continued to record various fluctuating concentrations of phosphate. Due to these fluctuations a different phosphate test made by LaMotte was obtained. This test was more consistent in the concentrations of phosphate that were found in the tanks. This test was used once, before adding more phosphates, to test the levels prior to adding them. This test was completed on November 19, 2019. After finding the levels were all about the same, with all the concentrations being at 8ppm and two treatment tanks being at 10 ppm, it was decided that more phosphate should be added to keep the treatment tanks at a higher concentration than the control tanks.

The second addition of phosphates was added on November 25, 2019. The goal was to attempt to get all the treatment levels to a 10-ppm concentration. The suggested number of milligrams per liter was 17.2 mg. The 1.72 grams of phosphate was added to 500 ml of clean natural water. The 1.72 grams was weighed out the same as the past amount of phosphate, by using a scalpel to scoop the sodium phosphate onto the tarred tray. After the correct amount of phosphate was obtained it was put in a beaker with 500 ml of clean natural water. About 20 ml of the 500 ml was used to wash the remaining sodium phosphate out of the tray and into the beaker. The beaker was then mixed on the Vortex Genie 2 for 7 minutes on level 7 until there was no

visible sodium phosphate. Then 20 ml of the 500 ml mixed solution was added to each treatment tank. To keep things consistent 20 ml of clean natural water was added to each control tank.

After adding the second addition of phosphates the water was tested for about two weeks. During the two weeks the phosphate levels ended up with all the treatments at 10 ppm except one, #4, that was at 20 ppm. Additionally, one of the control tanks, #1, was found to also be at 10 ppm. While the rest of the control tanks were at 8ppm. Because the one treatment tank was at 20 ppm and one control tank was at 10 ppm, it was decided that the treatment tanks would be increased from 10 ppm to 20 ppm. To do this the same mixture made on November 25, 2019 was created again on December 14, 2019. The only changes were that when vortexing the mixture to dissolve the sodium phosphate the Vortex Genie was set to level 9 and it was vortexing for only 5 minutes. Then 20 ml of the mixture were placed in all of the treatment tanks and 20 ml of clean natural water were placed in the control tanks.

On December 15, 2019 it was realized that the phosphate must be connected to calcium carbonate and was precipitating out of the water and into the gravel. In nature the phosphate level would be at 2-3 ppm. This meant that the controls were high due to excrement and food. The next goal was to attempt to get the controls at 3 ppm phosphate or lower. Charcoal and ammonia were added to the controls filters to help lower the phosphate level. Along with this testing for hardness or calcium carbonate began. To test the calcium carbonate levels an electronic reader was used. This system utilized a water sample and a test strip, the electronic reader then displays the level of calcium carbonate in the water. The hardness was tested from December 15, 2019-January 24, 2020 with a break in tests from December 18, 2019- January 12, 2020. During the break the salamanders were fed once a week by Dennis Ferraro. The hardness tests were replaced with pH tests when the hardness tests ran out of strips. The pH tests started

on January 31, 2020 and ended on March 13, 2020. The pH tests are done with an electronic device that measures the pH and temperature of the water it is inserted in. The phosphate levels, hardness levels, and pH levels can all be viewed in tables 1, 2, and 3 respectively.

It was decided on December 13, 2019 that there would be no more water added to the tanks and water that evaporated would not be replaced. This was done to increase the likelihood that the salamanders would morph into their terrestrial form. On January 13, 2020 after break the filters that were previously attached to the side of the tank were moved so that they were sitting in the bottom of the tank, so they could continue to filter the water properly. On January 17, 2020 the control filters were changed to help decrease the level of phosphate quicker. On February 19, 2020 it was decided that straight charcoal would be used in all of the control filters as the ammonia ran out. Two filters were placed in tank #1, a control tank, in an effort to bring the phosphate level from 10 ppm to 8ppm. Tank # 3, a treatment tank, had 20 ml of a phosphate solution added to it to bring the 10-ppm phosphate level up to 20 ppm. The phosphate solution was made according to the solution created on November 25th.

On February 21, 2020 it was found that the control tank #1 was still at 10 ppm. I tested the regular stream water on this date and found it to have a 0-ppm concentration of phosphate. It was then determined that 5 liters of the water in tank #1 would be removed and replaced with 5 liters of regular stream water in an effort to bring the phosphate level down. I also found that tank #3 was at 20 ppm which was the same as all the other treatment tanks. When removing the water from tank #1 the filter was unplugged and replugged in after the regular stream water was added.

On February 28, 2020 it was found that larvae #3 appeared to be almost completely done morphing, its' gills were small nubs on the sides of its' head. Additionally, larvae #7 gills

appeared to be shrinking. After testing the phosphate, it was found that control tanks #2, #5, and #7 had concentrations of phosphate at 10 ppm. Control tank #1 was at 8 ppm this was good as the week prior the water had been changed in an effort to decrease the phosphate levels and it worked. It was then determined that the other control tanks, #2, #5, and #7, would also have 5 liters of their water replaced in the same way as #1 to lower the concentration of phosphate below 10 ppm. The filters in tanks #3 and #4 were found to not be working on March 4, 2020. On March 6, 2020 the filters were fixed in tanks #3 and #4. On March 13, 2020 larvae #3 was found to have completed morphing completely and larvae #7 had gill nubs. Larvae #7 then received a lid to ensure that it did not escape its tank as did the other larvae that were already morphed. It was noted that some of the tank's water was below the filter line that allows the filter to properly work. These tanks were both control tanks so regular stream water was added to them with 3,000 liters being added to tank #5 and 1,000 liters being added to tank #1.

Analysis

Statistical analysis will be used to process the data. The temperature data should remain constant amongst the tanks. Additionally, the feeding data should not be significantly different amongst the individual salamanders. Phosphate data should remain the same amongst the control group and should be the same amongst the treatment group. The dates that the salamanders morphed will be compared amongst the treatment and non-treatment individuals to determine if there is a significant difference between them.

To test if phosphate significantly affected the metamorphosis of the larval salamanders all the data recorded was inputted into excel. A correlation coefficient was found and used to determine if there was significant data. The r value used to determine if there was significant data was + or - 0.7. It was also tested if temperature significantly affected the metamorphosis of

the larval salamanders. If the larvae had not yet morphed a 0 was inputted into the spreadsheet. On the day they had gill nubs they were considered morphed and a 1 was inputted into the spreadsheet.

Quality of the data was ensured by feeding the larvae roughly the same amount of food and keeping the room at a constant temperature. To test that the temperature did not change that much in relation to the tanks and that the feeding habits of the larvae did not change that much amongst each other correlation coefficients were tested amongst different pairs of the tanks.

Correlation coefficients were also found for the hardness and pH data to ensure that the control and treatment tanks were different from each other as a whole. Additionally, this data was used to ensure that treatment tanks had similar pH and hardness levels to other treatment tanks and vice versa with the control tanks.

Phosphate Levels (ppm) as Measured by LaMotte's Test Kit								
Date	Tank 1	Tank 2	Tank 3	Tank 4	Tank 5	Tank 6	Tank 7	Tank 8
19-Nov	8	8	8	10	8	10	8	8
26-Nov	10	8	10	10	8	8	8	10
3-Dec	10	8	8	10	8	8	8	8
12-Dec	10	8	10	20	8	10	8	10
15-Dec	10	10	10	10	10	10	8	10
13-Jan	10	10	10	10	8	10	8	10
7-Feb	10	8	10	20	8	20	8	20
14-Feb	8	8	10	20	8	20	8	20
21-Feb	10	8	20	20	10	20	8	20
28-Feb	8	10	30	20	10	20	10	20
4-Mar	n/a	8	n/a	n/a	6	n/a	8	n/a
6-Mar	10	8	30	20	6	20	8	20
13-Mar	6	10	30	20	8	30	10	20

Table 1: This table shows the measured phosphate levels in parts per million of the different tanks. These measurements were made with the third method of testing phosphate levels, LaMotte's test kit. If a box has a n/a that means a test was not done for that tank on that day.

Hardness ($CaCO_3$) Concentrations (ppm) as Measured by the eXact Strips								
Date	Tank 1	Tank 2	Tank 3	Tank 4	Tank 5	Tank 6	Tank 7	Tank 8
15-Dec	n/a	n/a	124	176	n/a	195	n/a	158
16-Dec	258	93	n/a	n/a	191	n/a	LO	n/a
13-Jan	137	123	LO	82	198	95	161	71
24-Jan	132	LO	LO	LO	232	10	90	LO

Table 2: This table shows the measured hardness concentrations in parts per million of the different tanks. A LO indicates that the concentration was too low to detect, and a n/a indicates no test was done for that tank on that day.

pH Concentrations as Measured by the Myron L. Company Ultrapen								
Date	Tank 1	Tank 2	Tank 3	Tank 4	Tank 5	Tank 6	Tank 7	Tank 8
31-Jan	9.9	10.35	10.4	9.59	8.23	8.72	9.89	8.73
7-Feb	7.04	9.24	7.18	7.32	8.85	8.04	8.76	7.79
14-Feb	8.2	8.05	6.52	6.38	6.86	6.84	6.76	7.08
21-Feb	2.31	6.12	6.83	5.68	5.85	7.31	7.39	6.72
28-Feb	3.05	7.1	6.1	7.26	7.17	6.56	7.06	6.66
4-Mar	7.57	7.84	7.19	6.96	7.11	6.98	7.08	6.88
6-Mar	4.86	4.81	6.47	6.76	6.19	6.28	6.83	6.47
13-Mar	2.94	7.11	6.88	6.84	6.86	6.4	6.84	6.65

Table 3: This table shows the pH level as measured by the Myron L. Company Ultrapen for each tank.

Results

All correlation coefficients were found using the =CORREL() function in excel spreadsheets. Table 4 shows the correlation coefficients relating the date the salamander morphed to the phosphate levels and the temperatures on those days. The only significant number found was larvae #3 in relation to the phosphate level. The r value found for larvae #3 was 0.9433701 indicating a positive correlation between the larvae morphing and the level of phosphate in the tank. It is noted that if the salamander did not morph a series of 0's was placed in the morphing column to be tested against the phosphate, and temperature variables. If the number in the morphing column did not change the correlation equation did not work, meaning there was no correlation between the two numbers.

There were only 4 larvae that morphed, they were #2, #3, #4, and #7. The reason #4 does not have a correlation coefficient for phosphate (PO_4) level is that the consistent phosphate tests did not begin until the day after it morphed so in the morphing column a series of 1's was placed. Because the numbers did not change in the morphing column the equation did not work. The equation did work for #4 in regard to the temperature because the temperature had been recorded before the larvae morphed, so the morphing column contained both 0's and 1's. When testing the correlation between the control tanks' and the treatment tanks' correlation coefficients it was found that both between the PO_4 level and the temperature had a r value of -1 as shown in Table 5. This -1 r value indicates that there is a strong negative correlation between the control and treatment tanks. This was supposed to be different as the phosphate levels were shown to differ especially towards the end of the research when the tests were more consistent.

When testing the correlation between the temperature of the different tanks it was found that all the tanks had a positive correlation of 0.97 and higher as shown in Table 6. This means that all the tanks were roughly the same temperature. The feeding habits of the different larvae

were also tested for correlation as they should have a significant positive r value which would show they were all eating about the same amount. This was reflected in the majority of the data shown in Table 7. However, the data also shows that larvae #4 had a significantly lower r value compared to the other larvae. The other larvae all had positive r values of 0.8 or higher which is what was expected. Larvae #4 had a positive r value of 0.2 or higher depending on which larvae it was associated with. Looking back at the eating habits recorded, there was a period of time after #4 morphed that it did not eat as much compared to the other larvae. It later picked its appetite back up and ate similarly to the other larvae.

When comparing the hardness ($CaCO_3$) of the water of each tank correlation coefficients were also used. It was expected that the hardness would be similar in the control tanks and similar in the treatment tanks, but as a group the control tanks and the treatment tanks would be different. The treatment tanks showed that they had similar hardness levels by the positive r value of 0.8 or higher shown in Table 8. The control tanks had an overall negative correlation with each other from 0.9 and higher. This was not as predicted; however, it is a possibility that the varying hardness levels were due to the differing amounts of ammonia and charcoal placed in the filters to attempt to lower the phosphate levels. Despite the variation within the control tanks overall the control tanks and treatment tanks had a negative correlation of about 0.6. The reason this number is not higher is most likely due to the variation within the control tanks.

When comparing the pH levels in the control and treatment tanks it was found that the two groups have a positive correlation with a r value of +1 as shown in Table 9. This indicates that both groups had similar pH's. Amongst the control group a positive r value of 0.7. While amongst the treatment groups they had a positive r value of 0.8 and higher.

Correlation Coefficients Related to Morphing

Tank #	PO_4 Level	Temperature
1	0	0
2	0.3651484	-0.6288942
3	0.9433701	0.2779605
4	0	-0.612262
5	0	0
6	0	0
7	0.6770032	-0.0136182
8	0	0

Table 4: Correlations comparing the phosphate levels to when the salamanders morphed and the temperature to the morphing data for each individual tank.

Correlation Between Morphed Control and Treatment		
PO4 Control	PO4 Treatment	PO4 Correlation
0.3651484	0.9433701	-1
0.6770032	0	
Temp control	Temp Treatment	Temp Correlation
-0.6288942	0.2779605	-1
-0.0136182	-0.612262	

Table 5: This data took the correlation coefficients from Table 1 and found the correlation coefficient between the control group and the treatment group for both the phosphate level and temperature.

Temp. Correlation Coefficients	
Correl 1&2	0.98886101
Correl 3&4	0.98480419
Correl 5&6	0.97946993
Correl 7&8	0.97785686

Table 6: This data is the correlation between different sets of tanks to compare their overall temperatures.

Feeding Correlation Coefficients	
Correl 1&2	0.87967595
Correl 3&4	0.25615987
Correl 5&6	0.91602829
Correl 7&8	0.8396011
Correl 2&3	0.80541665
Correl 4&5	0.37726518

Table 7: This data is the correlation between different sets of tanks to compare their overall eating habits.

$CaCO_3$ Correlation Control vs Treatment				
Control		Treatment		Correlation Control & Treatment
Correl 1&2	0.3170044	Correl 3&4	0.8850224	-0.6443463
Correl 5&7	-0.9170789	Correl 6&8	0.9999325	

Correl 1&7	-0.8820424	Correl 3&8	0.8937321	
Correl 2&5	-0.9224768	Correl 6&4	0.9999724	

Table 8: This data is the correlation between different sets of tanks to compare their hardness data obtained for each tank. In the third column is the correlation between the control and treatment groups.

pH Correlation Coefficient Control vs Treatment				
Control		Treatment		Correlation Control vs Treatment
Correl 1&2	0.74008456	Correl 3&4	0.85443239	1
Correl 5&7	0.75306331	Correl 6&8	0.94158538	

Table 9: This data is the correlation between different sets of tanks to compare their pH data obtained for each tank. In the third column is the correlation between the control and treatment groups.

Discussion

The main research question was if the elevated phosphates in the water would slow the metamorphosis. When comparing the time of metamorphosis to the level of phosphates in the water, phosphates were only shown to be significantly correlated with one of the larvae, #3. The data does not indicate if other treatment larvae that metamorphosed is also positively correlated with the increase in phosphate as the consistent phosphate tests began use the day after it had morphed. Overall the data rejects the hypothesis that the increased phosphate level slowed the metamorphosis rate, this is due to the fact that the larvae alternated between control and treatment when metamorphosing. The first larvae to morph was #4 followed by #2, then #3, and lastly #7.

Temperature was also compared to the metamorphosis rate. When comparing these data there was no significant data to be found in relation to supporting a correlation or disproving a correlation. This is to be expected as the temperature in the room the salamanders were in was kept constant, so each tank should be the same temperature +0.5 C. Meaning that there should not be much of a difference between the treatment and control tanks. This is shown in Table 3, with all the correlation coefficients being 0.9 or higher.

When comparing this study to my previous one regarding terrestrial salamanders it is hard to say if there are any major differences in the way the fertilizer acted to the terrestrial salamanders in comparison to the phosphate and larval salamanders. This is because each study did not have significant enough data to be able to back up any scientific claim. I will note that during the previous research using monoammonium fertilizer and terrestrial salamanders that there were 3 deaths and no deaths occurred with just the phosphate affecting the larval salamanders. Both areas of my research need to be studied further in order to be able to conclude whether or not the fertilizer or components of the fertilizer negatively impact the western barred tiger salamander.

The research done in this past year has been able to find a good method to measure phosphates in the water through LaMotte's test kit. Phosphate levels were also controlled by a combination of changing the water, changing filters and adding phosphate mixtures as necessary. If someone wanted to improve upon this study I would suggest using LaMotte's test kit to test the phosphate from the start to finish. I would also suggest adding the fertilizer sooner and possibly extending the length of the study to find out how long it would take all of the larvae to morph. Other studies have been done which show that nitrogen has a negative effect on salamanders, while there has not been many showing whether increased phosphate levels by

themselves affect the salamanders. This study adds to that knowledge which is not very numerous.

Although the study overall was inconclusive, there is data suggesting that the phosphate levels may influence the metamorphosis of the larval salamanders. Instead of what was hypothesized this data suggests that the influx of phosphate may have caused the metamorphosis of the larvae. However, with only one individual salamander suggesting this data, it is not significant enough to say that the phosphate level influences the metamorphosis of the larvae.

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